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A nonsense loss-of-function mutation in *PCSK1* contributes to dominantly inherited human obesity

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Abstract

Background: A significant proportion of severe familial forms of obesity remain genetically elusive. Taking advantage of our unique cohort of multi-generation obese families, we aimed to assess the contribution of rare mutations in 29 common obesity-associated genes to familial obesity, and to evaluate in these families the putative presence of nine known monogenic forms of obesity.

Methods: Through next-generation sequencing, we sequenced the coding regions of 34 genes involved in polygenic and/or monogenic forms of obesity in 201 participants (75 normal weight individuals, 54 overweight individuals and 72 individuals with obesity class I, II or III), from 13 French families. *In vitro* functional analyses were performed to investigate the mutation *PCSK1*-p.Arg80* which was identified in a family.

Results: A novel heterozygous nonsense variant in *PCSK1* (p.Arg80*), encoding a propeptide truncated to less than two exons (out of 14), was found to co-segregate with obesity in a three-generation family. We demonstrated that this mutation inhibits PCSK1 enzyme activity and that this inhibition most likely does not involve a strong physical interaction. Furthermore, both mutations *PCSK1*-p.Asn180Ser and *POMC*-p.Phe144Leu, which had previously been reported to be associated with severe obesity, were also identified in this study, but did not co-segregate with obesity. Finally, we did not identify any rare mutations co-segregating with obesity in common obesity susceptibility genes, except for *CADM2* and *QPCTL*, where we found two novel variants (p.Arg81His and p.Leu98Pro, respectively) in three obese individuals.

Conclusions: We showed for the first time that a nonsense mutation in *PCSK1* was likely to cause dominantly inherited human obesity, due to the inhibiting properties of the propeptide fragment encoded by the null allele. Furthermore, the present family sequencing design challenged the contribution of previously reported mutations to monogenic or at least severe obesity.

Introduction

Obesity has become one of the most important non-communicable pandemic diseases[1]. Many risk factors can explain this situation such as environmental causes but also strong genetic predispositions[1, 2]. Indeed, the heritability of body mass index (BMI) has been estimated to range between 40% and 70%[1, 2]. To date, BMI variation (or risk of common obesity) has been shown to be associated with more than 60 frequent single nucleotide polymorphisms (SNPs), which have mostly been identified by genome-wide association studies (GWAS)[3]. However, the overall contribution of these SNPs to BMI variation (or obesity risk) explains less than 15% of its heritability[3]. Recent studies have shown that rare coding mutations with strong effect and located in loci associated with type 2 diabetes or age-related macular degeneration (primarily identified by GWAS) may contribute to the missing heritability of these common diseases[4-7]. However, to our knowledge, such analyses have never been investigated in obesity so far.

On the other hand, rare coding mutations (or deletions) in nine genes (*BDNF*[8, 9], *LEP*[10, 11], *LEPR*[12], *MC4R*[13-15], *NTRK2*[16], *PCSK1*[17], *POMC*[18], *SH2B1*[19], *SIM1*[20, 21]) have been shown to cause severe early-onset obesity (mostly monogenic forms of obesity). Most of the proteins encoded by these genes play a key role in the leptin-melanocortin signaling pathway, which regulates appetite[1, 2]. Interestingly, frequent SNPs within (or close to) *BDNF*, *MC4R*, *PCSK1*, *POMC* and *SH2B1* have also been shown to contribute to common obesity risk[1-3, 22]. In addition, we can hypothesize that mutations in those genes may also co-segregate with obesity in families.

In the present study, we took advantage of our unique cohort of multi-generation obese families. We assessed the contribution of rare mutations in common obesity-associated genes to familial obesity in 13 large French pedigrees. Furthermore, we evaluated the putative presence of known monogenic forms of obesity in these families. For this purpose, we

sequenced 34 genes involved in polygenic and/or monogenic forms of obesity in 13 large French families, via next-generation sequencing (NGS).

Materials and methods

Patient selection

The study protocol was approved by all local ethics committees, and informed consent was obtained from each subject before participation in the study, in accordance with the Declaration of Helsinki principles. For children younger than 18 years, oral consent was obtained, and parents provided written informed consent.

The obese French adults and children were recruited either by the CNRS UMR8199 (Lille, France), by the Department of Nutrition of Hotel-Dieu Hospital (Paris, France) or by the *'Centre d'Etude du Polymorphisme Humain'* (CEPH, Saint-Louis hospital, Paris, France).

We selected 13 large French families including a total of 227 individuals with available clinical data, but 201 DNA samples satisfied quality and quantity criteria for NGS (**Table 1**). Large pedigrees including a proband whose the monogenic cause of obesity had been elucidated in previous studies were excluded from the selection[15, 20, 23, 24].

Obesity classes were described as following: normal weight, BMI < 25; overweight, 25 ≤ BMI < 30; obesity class I, 30 ≤ BMI < 35; obesity class II, 35 ≤ BMI < 40; obesity class III, 40 ≤ BMI.

Selection of the targeted genes

We selected a total of 34 genes: 29 genes that were found to be associated with BMI variation or risk of common obesity[1, 3], and nine genes that were found to be mutated in monogenic forms of obesity[2, 18-20] (**Table 2**).

Target enrichment and next-generation sequencing

Sequence capture of targeted regions (including exonic regions with a flanking 50 base pairs [bp] into each intron) was performed according to the manufacturer's protocol using SureSelect custom design reagent (Agilent Technologies, Santa Clara, CA, USA).

Briefly, 3µg of DNA was shorn by sonication (Bioruptor NGS, Diagenode, Liège, Belgium) and purified using Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA, USA). The fragment ends were repaired and adaptors were ligated to the fragments. The resulting DNA library was amplified by PCR and captured by hybridization to the biotinylated RNA library baits. Bound genomic DNA was purified with streptavidin coated magnetic Dynabeads (Invitrogen, Carlsbad, CA, USA) and re-amplified.

After capture, the enriched DNA samples were quantified using qPCR. Then, we performed 100bp paired-end (PE) read sequencing using the Illumina Genome Analyzer IIx (Illumina, San Diego, CA, USA), according to the manufacturer's recommendations.

Data analysis

For each run, demultiplexing of sequence data was performed with CASAVA (version 1.8.2, Illumina) to generate FASTQ files for each sample. Subsequently, sequence reads were mapped to the Human genome (hg19/GRC37) using the Burrows-Wheeler Aligner (BWA, version 0.6.1) to generate a BAM file. Variant calling was performed using SAMtools (version 0.1.18). The output VCF files were filtered using quality threshold of read depth $\geq 8\times$ and were annotated by extracting several features from Ensembl database: gene symbol and description, amino acid change, presence in dbSNP135, presence in 1000 Genomes and minor allele frequency in CEU population. Predictions of functional effects were also analyzed using dbNSFP[25] to integrate prediction scores from PolyPhen2[26], SIFT[27], MutationTaster[28] and LRT[29].

Sanger Sequencing

When putative causal variants were found, we confirmed their presence by Sanger sequencing via the 3730×l DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Primers were designed using VisualOMP. Primer sequences and PCR conditions can be provided upon

request. Sequencing reads were assembled and analyzed with Variant Reporter software (Applied Biosystems).

Cloning of constructs

Site directed mutagenesis was performed using the QuikChange II kit (Stratagene) using the FLAG-tagged *PCSK1* construct described previously as template[17]. The following mutations were introduced: p.Arg80*, p.Arg110*, p.Leu58Met, p.Leu58Met-p.Arg80*. Mutations were confirmed by Sanger sequencing.

Cell culture

HEK293T cells were plated in 3.4cm² or 10cm² dishes and transfected the next day with empty vector (pcDNA3; Invitrogen), human wild type *PCSK1*, *PCSK1*-p.Arg80* or *PCSK1*-pArg110* using Xtreme gene 9 transfection reagent (Roche Applied Science, Basel, Switzerland) according to the manufacturers protocol. Confluent wells were incubated with DMEM/F12 (Life Technologies) serum free medium overnight and conditioned medium was subsequently used for *in vitro* PCSK1 (or PC1/3) activity assay. Alternatively, cells were used for metabolic labeling.

Activity assay

PC1/3 enzymatic activity was assessed as previously described[30]. Briefly, 25µl conditioned medium from cells expressing different constructs was incubated in a total reaction volume of 50µl containing 0.2mM synthetic substrate Pyr-Glu-Arg-Thr-Lys-Arg-amino methylcoumarin (Bachem, Bubendorf, Switzerland), 5mM $CaCl_2$, 0.1M Na-acetate (pH 5.5), 0.1% Triton X-100, 10 µM E-64, 1 µM leupeptin, 10 µM pepstatin, and 100 µM tosyl phenylalanyl chloromethyl ketone (Sigma-Aldrich, St. Louis, MO). Each sample contained conditioned

medium from cells transfected with wild type PC1/3 combined with medium from cells transfected with different constructs as indicated in a ratio of 4:1.

Metabolic labeling

HEK293T cells were metabolically labeled as previously described[30]. Briefly, 10cm² dishes with HEK293T cells were transfected with empty vector, wild type *PCSK1*, *PCSK1*-p.Leu58Met and/or *PCSK1*-p.Leu58Met-p.Arg80*. *PCSK1* and *PCSK1*-p.Leu58Met contain a FLAG-tag between the propeptide and the catalytic domain. Transfected cells were metabolically labeled for 2h and chased for 1h. Co-immunoprecipitation experiments were performed with FlagM1 antibody (Sigma Aldrich, St. Louis, MO). FlagM1 only recognizes epitopes at a free amino terminus and will therefore only recognize PC1/3 cleaved carboxyterminal of RSKR-110.

Immunoblotting

Protein samples were prepared from lysate and conditioned medium from HEK293T cells expressing either wild type *PCSK1* with or without *PCSK1*-p.Arg80* or *PCSK1*-pArg110*. Wild type PC1/3 was detected with FlagM2 antibody (Sigma-Aldrich, St. Louis, MO), as previously described[17].

Results

Using NGS, we sequenced 34 genes involved in monogenic and/or polygenic obesity (**Table 2**) in 201 participants (including 75 normal weight individuals, 54 overweight individuals, 36 individuals with obesity class I, 18 individuals with obesity class II and 18 individuals with obesity class III), from 13 large French families with at least three generations (**Table 1**). Except for *FAIM1*, *POMC*, *QPCTL* and *SH2B1* genes, the median depth of coverage per gene was above 100× (**Table 2**).

We identified a total of 44 rare variants of interest (*i.e.* non-synonymous variants, essential splice-site variants, indels or nonsense variants with a minor allele frequency [MAF] below 1%), of which 22 were novel (**Table 3**). Among them, three non-synonymous variants were only carried by obese individuals (in a heterozygous state): p.Arg80* in *PCSK1* (three carriers in Family #11; **Table 3**), p.Arg81His in *CADM2* (two carriers in Family #9; **Table 3**) and p.Leu98Pro in *QPCTL* (one carrier in Family #4; **Table 3**).

The *PCSK1* p.Arg80* nonsense mutation was carried by patient #I2 with obesity class III, and by patients #II1 and #III1 who both presented with obesity class I (**Figure 1**). No normal weight subjects of Family #11 carried this mutation (**Figure 1**). However, the obese child #III3, sister of patient #III1, did not carry the mutation, thus she was likely to be a phenocopy (**Figure 1**). Of note, mutated patient #I2 with obesity class III also presented with type 2 diabetes (fasting plasma glucose [FPG] = 11 mmol/L) and her mutated son #II1 was pre-diabetic (FPG = 6.5 mmol/L). Episodes of diarrhea were not reported by the carriers, when we specifically asked them if they presented with malabsorption during their infancy. As the p.Arg80* nonsense mutation occurred at the beginning of *PCSK1* (encoding proprotein convertase PC1/3 which includes a total of 753 amino acids), the lack of function of the protein was very likely. In order to investigate whether the mutant propeptide fragment (encoded by *PCSK1*-p.Arg80*; **Figure 2a**) inhibits the wild type (WT) PC1/3 enzyme, an activity assay was performed using a fluorogenic substrate. The incubation of conditioned

medium containing both mutant propeptide fragment and WT PC1/3 decreased the activity of the latter by 20.0% (*P*=0.02, Student T test; **Figure 2b**). Of note, the inhibition was similar to inhibition of WT PC1/3 by the full-length propeptide of PC1/3 encoded by *PCSK1*-p.Arg110* (25.0%, *P*=0.006; **Figures 2a and 2b**), which has previously been shown to inhibit PC1/3[31]. Subsequently, we investigated the physical interaction between the mutant propeptide fragment (PC1/3-p.Arg80*) and WT PC1/3. For this purpose, we considered that PC1/3 propeptide (PC1/3-p.Arg110*) does not contain any methionines but that a methionine can be introduced at position 58 (p.Leu58Met) without interfering with normal protein maturation (**Figure 2a**)[30]. The full-length propeptide and the aminoterminal fragment of the cleaved propreptide co-immunoprecipitated with mature PC1/3-p.Leu58Met-p.Arg110, as previously shown[30], while we did not observed any co-immunoprecipitation between mutant PC1/3-p.Leu58Met-p.Arg80* and WT PC1/3 (**Figure 2c**). We subsequently investigated the maturation and secretion of WT PC1/3 in the presence or absence of PC1/3-p.Arg80* and the full-length propeptide of PC1/3 (PC1/3-p.Arg110*). Interestingly, we observed an increase in WT PC1/3 protein level in the presence of either complete or partial propeptide (PC1/3-p.Arg110* or PC1/3-p.Arg80*, respectively), indicating that the propeptide may retain WT PC1/3 in the endoplasmic reticulum or slows down intracellular transport (**Figure 2d**). Therefore, we demonstrated that the p.Arg80* nonsense mutation inhibits WT PC1/3 activity and that this inhibition most likely does not involve a strong physical interaction.

The *CADM2* p.Arg81His missense mutation was carried by an obese patient (BMI=31.6 at 69 years old) and her son presenting with obesity class II (BMI=37.4 at 43 years old; **Supplementary Figure 1**). This novel mutation was predicted to be probably deleterious according to dbNSFP (**Table 3**). Unfortunately, it was impossible to study a possible segregation of the mutation with obesity in Family #9 as the mother was outside the family and her son did not have any children (**Supplementary Figure 1**).

The *QPCTL* p.Leu98Pro missense mutation was carried by an adult presenting with obesity class III (BMI=52.0 at 60 years old). Her six brothers and sisters, with obesity, overweight or normal weight, did not carry this novel mutation, as well as her normal weight son (**Supplementary Figure 2**). Therefore, it was difficult to draw clear conclusions about this mutation, even if it was predicted to be deleterious according to dbNSFP (**Table 3**).

Interestingly, in the present study, we identified previously reported rare missense variants in *PCSK1* (p.Asn180Ser; **Table 3**)[23], *MC4R* (p.Ile251Leu; **Table 3**)[14] and *POMC* (p.Phe144Leu and p.Glu214Gly; **Table 3**)[32, 33], which were all present in the Human Gene Mutation Database (HGMD).

We previously reported the *PCSK1* p.Asn180Ser variant in two unrelated obese patients and we demonstrated its mild deleterious effect on the activity of the encoded protein, the proprotein convertase 1/3[23]. However, in the present study, this mutation was found in a normal weight adult (with a BMI of 20.2 at 42 years old) and his normal weight son (BMI=21.5 at 18 years old). The *PCSK1* p.Asn180Ser mutation was not identified in obese members of the same family, which lessened a marked contribution of this mutation to obesity in this family.

The rare *MC4R* p.Ile251Leu variant (rs52820871; MAF [European population] = 0.4%) was identified in an adult presenting with obesity class II (BMI=37.5 at 55 years old) and her non-obese son (BMI=25.7 at 32 years old) as well as her two normal weight daughters (BMI=24.7 at 31 years old and BMI=18.5 at 21 years old) in Family #6. These results are not in contradiction with our previous study which demonstrated a protective effect of the *MC4R* p.Ile251Leu variant in large cohorts[34]. The obesity of the mother was probably due to another genetic etiology.

The *POMC* p.Phe144Leu variant (rs201408477; MAF [European population] = 0.1%) was previously reported in one obese child and his obese father, and was shown to be strongly deleterious as this mutation dramatically altered POMC binding to MC4R[32]. In the present

study, the *POMC* p.Phe144Leu mutation was identified in three non-obese sisters (BMI=23.7, 23.9 and 29.0 at 24, 25 and 25 years old, respectively) from Family #1, lessening a significant contribution of this mutation to obesity in this family.

The *POMC* p.Glu214Gly variant (rs80326661; MAF [European population] = 0.3%) was previously reported in a 16.5 years old female obese adolescent who also carried an insertion and a nonsense mutation in the same gene (in a heterozygous state)[33]. In the present study, the heterozygous mutation was carried by a normal weight adult (BMI=24.9 at 76 years old), and was transmitted to her obese daughter (BMI=43.0 at 48 years old) and to her normal weight granddaughter (BMI Z-score=0.27 at 14 years old) in Family #7, lessening a strong contribution of this mutation to obesity in this family.

Discussion

In the present study, by assessing the contribution of rare mutations to common or monogenic obesity-associated genes in 201 individuals, from 13 large French pedigrees with familial obesity, we identified a novel nonsense mutation (c.238C>T / p.Arg80*) in *PCSK1* (in a heterozygous state) which is likely to cause obesity in a three-generation family.

PCSK1 encodes the proprotein convertase subtilisin/kexin type 1 (also known as proprotein convertase PC1/3) which is considered the major processing enzyme of prohormones (including proinsulin, proopiomelanocortin, proglucagon, prorenin, progastrin, progonadotrophin releasing hormone…) involved in a broad of endocrine, enteroendocrine and neuronal pathways[23, 35]. Genetic association studies in large cohorts showed that common single nucleotide polymorphisms (SNPs) in *PCSK1* contributed to increased risk of obesity[22] and to variation of both fasting proinsulin levels and fasting plasma glucose levels[36]. All these associations were highly significant but harbored a weak effect, which was typical for common SNPs. In contrast, rare congenital deficiency of *PCSK1* has been reported so far in less than 20 unrelated probands (carrying homozygous or compound heterozygous mutations) who presented with malabsorptive diarrhea, failure to thrive during early infancy associated with high mortality rate, severe early onset obesity, polyphagia, central diabetes insipidus, hypogonadism, hyperproinsulinemia and other endocrine dysfunctions[17, 30, 37-39]. Notably, it appeared that when congenital carriers of *PCSK1* mutations experienced severe failure to thrive during early infancy because of persistent malabsorption, they only presented with moderate obesity during late childhood and adolescence[39]. It could be explained by the homozygosity of highly deleterious variants (including nonsense or frameshift mutations) carried by these patients[39]. Martín and colleagues hypothesized that when the recessive mutations are milder, the carriers present with a more profound obesity[39]. Furthermore, we previously reported a significant enrichment of rare heterozygous variants in *PCSK1* causing partial PCSK1 deficiency, in unrelated obese individuals[23]. However, the vertical transmission

of these mild loss-of-function variants was not investigated in families and they were not fully penetrant (with an odds ratio of ~8)[23]. In the present study, we showed that a nonsense heterozygous mutation (p.Arg80*) at the beginning of *PCSK1* co-segregated with obesity in three generations of a French family, following a dominant mode of inheritance. Of note, the propeptide of PC1/3 (PC1/3-p.Arg110*; **Figure 2a**) functions as an intramolecular chaperone, essential for folding and transport through the secretory pathway. The propeptide is cleaved at position 110 in the endoplasmic reticulum by an intramolecular process[40]. However, in the p.Arg80* mutation carriers, the propeptide remains associated with PC1/3 till the late Golgi compartments where a second cleavage at position 80 occurs and the two fragments dissociate from PC1/3[41]. The effect of the propeptide *in trans* has been shown to act as a slow tight-binding inhibitor of the native enzyme[31]. The main inhibitory potency of the propeptide has been attributed to the carboxy-terminal region of the prodomain[42, 43]. Here, we demonstrated that the amino-terminal propeptide, only containing the sequence up till the cleavage site at position 80, was able to inhibit WT PC1/3 activity and that this inhibition most likely does not involve a strong physical interaction. The observation that both amino-terminal propeptide (PC1/3-p.Arg80*) and the full-length propeptide (PC1/3-p.Arg110*) increase the amount of intracellular WT PC1/3 is a novel finding which suggests that in addition to the inhibition, the propeptide has additional effects on PC1/3 trafficking and stability. Therefore, the mutation found in the family does not only inactivate one *PCSK1* allele, but its gene product also interferes with the functioning of the gene product of the unaffected allele. This is a likely explanation for the dominant inheritance of the obesity phenotype. However, the inhibition of PC1/3 is only partial which would explain why no multihormonal disorder is observed in these patients, in contrast to the phenotype of *PCSK1* null patients[39]. To our knowledge, it is the first demonstration that partial haploinsufficiency of *PCSK1* can cause a dominant form of familial obesity.

In summary, *PCSK1* can harbor different genetic events with various frequencies, which are associated with a wide range of phenotypes: 1/ rare null mutations causing congenital PCSK1 deficiency, which lead to diarrhea, failure to thrive, early onset obesity and a plethora of other severe clinical features[17, 30, 37-39]; 2/ a rare nonsense loss-of-function mutation at the heterozygous state, causing a dominant form of mendelian familial obesity associated with glucose intolerance; 3/ partial loss-of-function heterozygous mutations conferring high risk for common obesity[23]; and finally 4/ frequent SNPs associated with a mild increase in risk of common obesity, and modest variations of both fasting proinsulin levels and fasting plasma glucose levels[22, 36].

With regard to the other genes involved in monogenic obesity, we did not identify any putatively causal mutations leading to obesity in the 12 remaining families. Notably, our family sequencing design lessened the marked contribution of previously reported mutations in *PCSK1* (p.Asn180Ser)[23] or *POMC* (p.Phe144Leu)[32] to monogenic or at least severe obesity. It shows the power of family study to evaluate the true contribution of rare mutations, even in excellent candidate genes, to disease risk.

Finally, we did not find any rare mutations co-segregating with obesity in common obesity susceptibility genes, except for two GWAS identified genes (*CADM2* and *QPCTL*) which may deserve further genetic investigations. No rare coding mutations in these two genes have been listed in the HGMD database. *CADM2* encodes the cell adhesion molecule 2, which belongs to the immunoglobulin superfamily and plays a key role in the cytoskeleton, while *QPCTL* encodes the glutaminyl-peptide cyclotransferase-like. Expression of both of these genes was shown to be modified in cancers[44, 45].

In conclusion, we identified a novel nonsense mutation in *PCSK1* in the heterozygous state which caused obesity in three generations from a French family. We did not find any other rare mutations (among the list of sequenced genes) co-segregating with obesity in the 12 remaining families, which may be surprising. We could imagine two possibilities: 1/ the

causal mutations are elsewhere in the genome and thus we should perform whole-exome sequencing or whole-genome sequencing to find them; 2/ the obesity in these families is polygenic and depends on multiple genetic and epigenetic variants in interaction (or not) with the nurture.

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Conflict of Interest

The authors declare no competing financial interests.

Supplementary information

Supplementary information is available at International Journal of Obesity's website.

References

1. El-Sayed Moustafa JS, Froguel P. From obesity genetics to the future of personalized obesity therapy. *Nature reviews. Endocrinology* 2013; **9**(7)**:** 402-413.

2. Ramachandrappa S, Farooqi IS. Genetic approaches to understanding human obesity. *The Journal of clinical investigation* 2011; **121**(6)**:** 2080-2086.

3. Berndt SI, Gustafsson S, Mägi R, Ganna A, Wheeler E, Feitosa MF *et al.* Genome-wide meta-analysis identifies 11 new loci for anthropometric traits and provides insights into genetic architecture. *Nature genetics* 2013; **45**(5)**:** 501-512.

4. Bonnefond A, Clément N, Fawcett K, Yengo L, Vaillant E, Guillaume J-L *et al.* Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nature genetics* 2012; **44**(3)**:** 297-301.

5. Helgason H, Sulem P, Duvvari MR, Luo H, Thorleifsson G, Stefansson H *et al.* A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nature genetics* 2013.

6. Seddon JM, Yu Y, Miller EC, Reynolds R, Tan PL, Gowrisankar S *et al.* Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nature genetics* 2013.

7. Zhan X, Larson DE, Wang C, Koboldt DC, Sergeev YV, Fulton RS *et al.* Identification of a rare coding variant in complement 3 associated with age-related macular degeneration. *Nature genetics* 2013.

8. Gray J, Yeo GSH, Cox JJ, Morton J, Adlam A-LR, Keogh JM *et al.* Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. *Diabetes* 2006; **55**(12)**:** 3366-3371.

9. Han JC, Liu Q-R, Jones M, Levinn RL, Menzie CM, Jefferson-George KS *et al.* Brain-derived neurotrophic factor and obesity in the WAGR syndrome. *The New England journal of medicine* 2008; **359**(9)**:** 918-927.

10. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ *et al.* Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 1997; **387**(6636)**:** 903-908.

11. Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD. A leptin missense mutation associated with hypogonadism and morbid obesity. *Nature genetics* 1998; **18**(3)**:** 213-215.

12. Clément K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D *et al.* A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998; **392**(6674)**:** 398-401.

13. Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G *et al.* Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *The Journal of clinical investigation* 2000; **106**(2)**:** 271-279.

14. Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P. Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *The Journal of clinical investigation* 2000; **106**(2)**:** 253-262.

15. Vaisse C, Clement K, Guy-Grand B, Froguel P. A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nature genetics* 1998; **20**(2)**:** 113-114.

16. Yeo GSH, Connie Hung C-C, Rochford J, Keogh J, Gray J, Sivaramakrishnan S *et al.* A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. *Nature neuroscience* 2004; **7**(11)**:** 1187-1189.

17. Jackson RS, Creemers JW, Ohagi S, Raffin-Sanson ML, Sanders L, Montague CT *et al.* Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nature genetics* 1997; **16**(3)**:** 303-306.

18. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Grüters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nature genetics* 1998; **19**(2)**:** 155-157.

19. Doche ME, Bochukova EG, Su H-W, Pearce LR, Keogh JM, Henning E *et al.* Human SH2B1 mutations are associated with maladaptive behaviors and obesity. *The Journal of clinical investigation* 2012; **122**(12)**:** 4732-4736.

20. Bonnefond A, Raimondo A, Stutzmann F, Ghoussaini M, Ramachandrappa S, Bersten DC *et al.* Loss-of-function mutations in SIM1 contribute to obesity and Prader-Willi-like features. *The Journal of clinical investigation* 2013; **123**(7)**:** 3037-3041.

21. Ramachandrappa S, Raimondo A, Cali AMG, Keogh JM, Henning E, Saeed S *et al.* Rare variants in single-minded 1 (SIM1) are associated with severe obesity. *The Journal of clinical investigation* 2013; **123**(7)**:** 3042-3050.

22. Benzinou M, Creemers JWM, Choquet H, Lobbens S, Dina C, Durand E *et al.* Common nonsynonymous variants in PCSK1 confer risk of obesity. *Nature genetics* 2008; **40**(8)**:** 943-945.

23. Creemers JWM, Choquet H, Stijnen P, Vatin V, Pigeyre M, Beckers S *et al.* Heterozygous mutations causing partial prohormone convertase 1 deficiency contribute to human obesity. *Diabetes* 2012; **61**(2)**:** 383-390.

24. Stutzmann F, Tan K, Vatin V, Dina C, Jouret B, Tichet J *et al.* Prevalence of melanocortin-4 receptor deficiency in Europeans and their age-dependent penetrance in multigenerational pedigrees. *Diabetes* 2008; **57**(9)**:** 2511-2518.

25. Liu X, Jian X, Boerwinkle E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. *Human mutation* 2011; **32**(8)**:** 894-899.

26. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P *et al.* A method and server for predicting damaging missense mutations. *Nature methods* 2010; **7**(4)**:** 248-249.

27. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic acids research* 2003; **31**(13)**:** 3812-3814.

28. Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nature methods* 2010; **7**(8)**:** 575-576.

29. Chun S, Fay JC. Identification of deleterious mutations within three human genomes. *Genome research* 2009; **19**(9)**:** 1553-1561.

30. Farooqi IS, Volders K, Stanhope R, Heuschkel R, White A, Lank E *et al.* Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3. *The Journal of clinical endocrinology and metabolism* 2007; **92**(9)**:** 3369-3373.

31. Fugere M, Limperis PC, Beaulieu-Audy V, Gagnon F, Lavigne P, Klarskov K *et al.* Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. *The Journal of biological chemistry* 2002; **277**(10)**:** 7648-56.

32. Dubern B, Lubrano-Berthelier C, Mencarelli M, Ersoy B, Frelut M-L, Bouglé D *et al.* Mutational analysis of the pro-opiomelanocortin gene in French obese children led to the identification of a novel deleterious heterozygous mutation located in the alpha-melanocyte stimulating hormone domain. *Pediatric research* 2008; **63**(2)**:** 211-216.

33. Hinney A, Becker I, Heibült O, Nottebom K, Schmidt A, Ziegler A *et al.* Systematic mutation screening of the pro-opiomelanocortin gene: identification of several genetic variants including three different insertions, one nonsense and two missense point

mutations in probands of different weight extremes. *The Journal of clinical endocrinology and metabolism* 1998; **83**(10)**:** 3737-3741.

34. Stutzmann F, Vatin V, Cauchi S, Morandi A, Jouret B, Landt O *et al.* Non-synonymous polymorphisms in melanocortin-4 receptor protect against obesity: the two facets of a Janus obesity gene. *Human molecular genetics* 2007; **16**(15)**:** 1837-1844.

35. Seidah NG. The proprotein convertases, 20 years later. *Methods in molecular biology (Clifton, N.J.)* 2011; **768:** 23-57.

36. Strawbridge RJ, Dupuis J, Prokopenko I, Barker A, Ahlqvist E, Rybin D *et al.* Genome-wide association identifies nine common variants associated with fasting proinsulin levels and provides new insights into the pathophysiology of type 2 diabetes. *Diabetes* 2011; **60**(10)**:** 2624-2634.

37. Frank GR, Fox J, Candela N, Jovanovic Z, Bochukova E, Levine J *et al.* Severe obesity and diabetes insipidus in a patient with PCSK1 deficiency. *Molecular genetics and metabolism* 2013; **110**(1-2)**:** 191-194.

38. Jackson RS, Creemers JWM, Farooqi IS, Raffin-Sanson M-L, Varro A, Dockray GJ *et al.* Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *The Journal of clinical investigation* 2003; **112**(10)**:** 1550-1560.

39. Martín MG, Lindberg I, Solorzano-Vargas RS, Wang J, Avitzur Y, Bandsma R *et al.* Congenital proprotein convertase 1/3 deficiency causes malabsorptive diarrhea and other endocrinopathies in a pediatric cohort. *Gastroenterology* 2013; **145**(1)**:** 138-148.

40. Goodman LJ, Gorman CM. Autoproteolytic activation of the mouse prohormone convertase mPC1. *Biochemical and biophysical research communications* 1994; **201**(2)**:** 795-804.

41. Rabah N, Gauthier D, Wilkes BC, Gauthier DJ, Lazure C. Single amino acid substitution in the PC1/3 propeptide can induce significant modifications of its inhibitory profile toward its cognate enzyme. *The Journal of biological chemistry* 2006; **281**(11)**:** 7556-67.

42. Basak A, Koch P, Dupelle M, Fricker LD, Devi LA, Chretien M *et al.* Inhibitory specificity and potency of proSAAS-derived peptides toward proprotein convertase 1. *The Journal of biological chemistry* 2001; **276**(35)**:** 32720-8.

43. Basak A, Lazure C. Synthetic peptides derived from the prosegments of proprotein convertase 1/3 and furin are potent inhibitors of both enzymes. *The Biochemical journal* 2003; **373**(Pt 1)**:** 231-9.

44. He W, Li X, Xu S, Ai J, Gong Y, Gregg JL *et al.* Aberrant methylation and loss of CADM2 tumor suppressor expression is associated with human renal cell carcinoma tumor progression. *Biochemical and biophysical research communications* 2013; **435**(4)**:** 526-532.

45. Kehlen A, Haegele M, Menge K, Gans K, Immel U-D, Hoang-Vu C *et al.* Role of glutaminyl cyclases in thyroid carcinomas. *Endocrine-related cancer* 2013; **20**(1)**:** 79-90.

46. Park WD, O'Brien JF, Lundquist PA, Kraft DL, Vockley CW, Karnes PS *et al.* Identification of 58 novel mutations in Niemann-Pick disease type C: correlation with biochemical phenotype and importance of PTC1-like domains in NPC1. *Human mutation* 2003; **22**(4)**:** 313-325.

Table 1. Characteristics of the 13 French families

Numbers in square brackets shows the number of sequenced DNA samples

Table 2. List of the targeted genes and sequence quality

Table 3. List of rare variants identified in the targeted genes (with a MAF below 1%)

D, deleterious; ***PD***, probably deleterious; ***PH***, probably harmless; ***NA***, not available; ***SNP***, single nucleotide polymorphism

*MAF according to the public database dbSNP137

***In silico* prediction effect of each variant according to dbNSFP (see **Methods**)

Figure 1. Pedigree of Family #11 showing obesity status of each member, as well as genetic status, birth date, age at examination and BMI

NM denotes the presence of the heterozygous *PCSK1* p.Arg80* mutation and NN denotes the absence of mutation at the same locus.

Circles represent female participants and squares male participants.

Under each participant: genetic status, year of birth, age at examination, BMI (zBMI only for children).

Figure 2. **Mutant propeptide PC1/3-Arg80* inhibits WT PC1/3 *in vitro.***

a) Schematic representation of the propeptide. N-terminal propeptide (PC1/3-Arg80*) has a predicted molecular weight of 6.2kDa and the full-length propeptide (PC1/3-Arg110*) has a predicted molecular weight of 12.8kDa.

b) PC1/3 activity was measured using a fluorogenic substrate and conditioned medium from HEK293T cells transfected with WT PC1/3 together with conditioned medium of cells expressing different constructs as indicated above the figure. Control samples contained only medium from cells transfected with empty vector (EV). Activity was normalized to WT PC1/3 activity. Error bars represent mean ±SEM, n=5, *P<0.05, **P<0.01.

c) Co-immunoprecipitation of the mutant propeptide PC1/3-p.Arg80* with WT PC1/3 enzyme. To specifically label the mutant propeptide, a methionine was introduced at position 58 (p.Leu58Met).

d) Western blot of lysate and methanol precipitated medium from HEK293T cells expressing WT with or without co-expression of propeptide constructs. A representative image is shown (n=4).

Table 1. Characteristics of the 13 French families

Family	Number of generations	Number of relatives (Male:Female)	Number of relatives presenting with:				
			Normal weight	Overweight	Obesity Class I	Obesity Class II	Obesity Class III
1	4	22 [18] (9 [5]:13 [13])	8 [8]	5 [5]	1 [1]	2 [0]	6 [4]
2	4	18 [14] (8 [4]:10 [10])	4 [2]	5 [5]	3 [2]	3 [3]	3 [2]
3	3	14 [9] (6 [4]:8 [5])	6 [5]	4 [3]	2 [0]	2 [1]	0 [0]
4	3	15 [13] (5 [4]:10 [9])	7 [7]	3 [1]	4 [4]	0 [0]	1 [1]
5	3	15 [12] (7 [7]:8 [5])	6 [5]	1 [1]	6 [4]	2 [2]	0 [0]
6	3	14 [12] (5 [4]:9 [8])	6 [4]	2 [2]	3 [3]	3 [3]	0 [0]
7	3	15 [15] (5 [5]:10 [10])	8 [8]	2 [2]	2 [2]	2 [2]	1 [1]
8	3	20 [17] (6 [5]:14 [12])	9 [6]	4 [4]	3 [3]	1 [1]	3 [3]
9	4	18 [18] (8 [8]:10 [10])	1 [1]	7 [7]	6 [6]	3 [3]	1 [1]
10	4	29 [28] (12 [12]:17 [16])	9 [9]	10 [10]	4 [4]	0 [0]	6 [5]
11	3	10 [10] (4 [4]:6 [6])	4 [4]	2 [2]	3 [3]	0 [0]	1 [1]
12	3	13 [11] (6 [4]:7 [7])	3 [3]	4 [4]	4 [2]	2 [2]	0 [0]
13	3	24 [24] (9 [9]:15 [15])	13 [13]	8 [8]	2 [2]	1 [1]	0 [0]

Table 2. List of the targeted genes and sequence quality

Gene	Transcript ID	Gene location	Monogenic obesity	Common obesity	Median depth of coverage [lower quartile - upper quartile]
BDNF	NM_001143805.1	11p13	×	×	291 [171 - 411]
CADM2	NM_001167674.1	3p12.1		×	218 [77 - 352]
ETV5	NM_004454.2	3q28		×	205 [124 - 311]
FAIM2	NM_012306.2	12q13		×	87 [45 - 136]
FTO	NM_001080432.2	16q12.2		×	241 [127 - 347]
GNPDA2	NM_138335.1	4p12		×	270 [189 - 347]
GPRC5B	NM_016235.1	16p12		×	228 [99 - 364]
KCTD15	NM_001129994.1	19q13.11		×	130 [64 - 240]
LEP	NM_000230.2	7q31.3	×		201 [78 - 293]
LEPR	NM_002303.5	1p31	×		343 [222 - 445]
LRP1B	NM_018557.2	2q21.2		×	281 [192 - 377]
MAF	NM_005360.4	16q22-q23		×	234 [74 - 379]
MAP2K5	NM_145160.2	15q23		×	116 [66 - 241]
MC4R	NM_005912.2	18q22	×	×	341 [260 - 419]
MTCH2	NM_014342.3	11p11.2		×	243 [143 - 359]
MTIF3	NM_152912.4	13q12.2		×	209 [56 - 339]
NEGR1	NM_173808.2	1p31.1		×	339 [221 - 428]
NPC1	NM_000271.4	18q11.2		×	221 [128 - 334]
NRXN3	NM_004796.5	14q31		×	287 [184 - 398]
NTRK2	NM_006180.3	9q22.1	×		306 [166 - 421]
NUDT3	NM_006703.3	6p21.2		×	243 [121 - 357]
PCSK1	NM_000439.4	5q15-q21	×		280 [161 - 393]
POMC	NM_000939.2	2p23.3	×	×	42 [16 - 101]
PRKD1	NM_002742.2	14q11		×	259 [155 - 374]
PTBP2	NM_021190.2	1p21.3		×	326 [206 - 422]
QPCTL	NM_017659.3	19q13.32		×	69 [41 - 115]
SEC16B	NM_033127.2	1q25.2		×	188 [99 - 313]
SH2B1	NM_001145795.1	16p11.2	×	×	63 [35 - 105]
SIM1	NM_005068.2	6q16.3	×		318 [141 - 451]
SLC39A8	NM_001135146.1	4q22-q24		×	232 [67 - 370]
TFAP2B	NM_003221.3	6p12		×	225 [108 - 352]
TMEM18	NM_152834.2	2p25.3		×	157 [78 - 278]
TNKS	NM_003747.2	8p23.1		×	272 [167 - 403]
TNNI3K	NM_015978.2	1p31.1		×	264 [140 - 402]

Table 3. List of rare variants identified in the targeted genes (with a MAF below 1%)

Gene	Variant	Known SNP	MAF*	Heterozygous carriers			Number of affected families	Predicted severity**	Ref
				Obese	Over-weight	Normal weight			
CADM2	c.248G>A / p.Arg81His	NA	NA	2	0	0	1	PD	NA
FAIM2	c.287A>G / p.Gln96Arg	rs149184489	NA	2	1	1	2	PD	NA
FAIM2	c.827T>A / p.Leu276Gln	NA	NA	2	2	0	1	PD	NA
FTO	c.767G>A / p.Ser256Asn	rs144743617	0.001	0	0	2	1	PD	NA
FTO	c.1418G>A / p.Arg473Gln	NA	NA	2	1	1	1	PD	NA
LEPR	c.611A>G / p.Lys204Arg	rs146442768	NA	1	1	0	1	PH	NA
LRP1B	c.1379G>A / p.Arg460Gln	NA	NA	0	0	1	1	PD	NA
LRP1B	c.4856G>T / p.Arg1619Leu	NA	NA	1	4	0	1	PD	NA
LRP1B	c.7366G>A / p.Val2456Ile	rs150174082	0.001	0	0	2	1	PD	NA
LRP1B	c.7420G>A / p.Gly2474Ser	rs146867394	0.005	4	3	2	4	PH	NA
LRP1B	c.9309G>C / p.Trp3103Cys	NA	NA	1	0	1	1	PD	NA
LRP1B	c.9532G>A / p.Ala3178Thr	rs72899872	0.007	2	1	2	1	PD	NA
LRP1B	c.12047C>T / p.Pro4016Leu	rs150957163	0.005	2	2	2	2	PD	NA
LRP1B	c.12161A>C / p.Glu4054Ala	rs79879036	0.003	3	0	1	1	PD	NA
MAF	c.21G>T / p.Met7Ile	NA	NA	0	1	0	1	PD	NA
MC4R	c.512G>T / p.Ser171Ile	NA	NA	0	0	1	1	PD	NA
MC4R	c.751A>C / p.Ile251Leu	rs52820871	0.004	1	1	2	1	PD	14
MTCH2	c.357C>G / p.His119Gln	rs75020795	0.001	0	1	0	1	PD	NA
MTCH2	c.899A>G / p.Lys300Arg	rs73465610	NA	1	2	1	3	PH	NA
MTIF3	c.413A>G / p.Gln138Arg	rs140262959	0.007	2	1	3	3	D	NA
NEGR1	c.94C>G / p.Leu32Val	rs142674139	0.003	3	1	1	1	PH	NA
NPC1	c.665A>G / p.Asn222Ser	rs55680026	0.001	0	0	1	1	PD	46
NPC1	c.2429T>G / p.Val810Gly	NA	NA	6	4	2	7	D	NA
NPC1	c.2509A>G / p.Ile837Val	NA	NA	0	0	2	1	D	NA
NRXN3	c.924G>C / p.Arg308Ser	rs138594346	NA	0	0	3	1	PD	NA
NRXN3	c.1219C>T / p.Arg407Trp	rs144412380	NA	0	0	1	1	PD	NA
NRXN3	c.2311C>A / p.Leu771Ile	NA	NA	1	1	2	1	PD	NA
PCSK1	c.238C>T / p.Arg80*	NA	NA	3	0	0	1	PD	NA
PCSK1	c.539A>G / p.Asn180Ser	NA	NA	0	0	2	1	PD	23

POMC	c.430T>C / p.Phe144Leu	rs201408477	0.001	0	1	2	1	PD	[32]
POMC	c.641A>G / p.Glu214Gly	rs80326661	0.003	1	0	2	1	PD	[33]
PRKD1	c.20T>C / p.Leu7Pro	NA	NA	2	0	1	2	PD	NA
PTBP2	c.95T>G / p.Met32Arg	NA	NA	0	0	2	1	PD	NA
PTBP2	c.1010A>G / p.Asn337Ser	NA	NA	0	0	1	1	PD	NA
QPCTL	c.293T>C / p.Leu98Pro	NA	NA	1	0	0	1	D	NA
QPCTL	c.554C>T / p.Thr185Met	NA	NA	0	0	2	1	PD	NA
SH2B1	c.1156C>A / p.Arg386Ser	NA	NA	2	2	2	1	PH	NA
SH2B1	c.1636A>G / p.Thr546Ala	NA	NA	0	0	3	1	D	NA
SH2B1	c.1988C>T / p.Ala663Val	rs190981290	0.003	0	0	1	1	PD	NA
SIM1	c.206G>C / p.Arg69Pro	NA	NA	1	1	0	1	PD	NA
SLC39A8	c.745C>G / p.Pro249Ala	rs147912552	0.002	0	1	0	1	PH	NA
TNKS	c.58C>T / p.Pro20Ser	NA	NA	0	1	0	1	PH	NA
TNKS	c.145A>G / p.Thr49Ala	rs201993870	NA	5	0	2	2	PD	NA
TNKS	c.410C>A / p.Ser137Tyr	NA	NA	0	1	0	1	PD	NA